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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING RAD51

(57) Abstract: Described herein are methods for inhibiting Rad51 activity by the administration of a Rad51 inhibitor. The Rad51 inhibitors described herein are small molecules, preferably smaller than 4 kd.

METHODS AND COMPOSITIONS FOR INHIBITING RAD51

FIELD OF THE INVENTION

The invention relates to methods and compositions including small molecules for inhibiting RAD51.

BACKGROUND OF THE INVENTION

Homologous recombination is a fundamental process important for creating genetic diversity and maintaining genome integrity. In *E. coli*, RecA protein plays a central role in homologous recombination. *In vitro*, RecA protein promotes homologous pairing of double-stranded DNA with single-stranded DNA or partially single-stranded DNA molecules (Radding, *Genetic Recombination*. Washington, American Society for Microbiology, pp 193-230 (1988); Radding, *J. Biol. Chem.* **266**:5355-5358 (1991); Kowalczykowski & Eggleston, *Annu. Rev. Biochem.* **63**:991-1043 (1994)). Recently, genes homologous to *E. coli* recA and yeast rad51 were isolated from all groups of eukaryotes, including mammals (Morita, et al., *Proc. Natl. Acad. Sci. USA* **90**:6577-6580 (1993); Shinohara, et al., *Nature Genet.* **4**:239-243 (1993); Heyer, *Experentia* **50**:223-233 (1994); Maeshima, et al., *Gene* **160**:195-200 (1995)). Phylogenetic analysis by Ogawa and co-workers suggest there are two sub-families within the eukaryotic RecA homologous: the Rad51-like genes (Rad51 of human, mouse, chicken, *S. cerevisiae, S. pombe* and Mei3 of *Neurospora crassa*) and the Dmcl-like genes (*S. cerevisiae*, Dmcl and *Lilium longiflorum* LIM15) (Ogawa, et al., *Science* **259**:1896-1899 (1993)). All Rad51

genes share significant homology with residues 33-240 of *E. coli* RecA protein, which has been identified as a 'homologous core' region.

Although the yeast rad51 gene was cloned and sequenced (Basile et al., *Mol. Cell. Biol.* **12**:3235-3246 (1992); Aboussekhara, et al., *Mol. Cell. Biol.* **12**:3224-3234 (1992)) several years ago, it was not initially thought to be a homologue of RecA, because the sequence homology between RecA and Rad51 is limited. The true extent of structural conservation between RecA protein and Rad51 protein became apparent only after the discovery that yeast Rad51 protein forms nucleoprotein filaments nearly identical to the nucleoprotein filaments formed by RecA protein (Ogawa, et al., *CSH Symp. Quant. Biol.* **58**:567-576 (1993); Story et al., *Science* **259**:1892-1896 (1993)).

Yeast and human Rad51 proteins have been purified and characterized biochemically. Like *E. coli* RecA protein, yeast and human Rad51 protein polymerizes on single-stranded DNA to form a right-handed helical nucleoprotein filament which extends DNA by 1.5 times its original length (Story, et al., *Science* **259**:1892-1896 (1993); Benson, et al., *EMBO J.* **13**:5764-5771 (1994)). Moreover, similar to RecA protein, Rad51 protein promotes homologous pairing and strand exchange in an ATP dependent reaction (Sung, *Science* **265**:1241-1243 (1994); Baumann, et al., *Cell* **87**:757-766 (1996); Gupta, et al., *Proc. Natl. Acad. Sci. USA* **94**:463-468 (1997)).

Rad51 protein is important for the repair of double-strand breaks in damaged cells. In *S. cerevisiae*, genes with homology to recA include Rad51, Rad57 and Dmcl. Rad51 is a member of the Rad52 epistasis group, which includes Rad50, Rad51, Rad52, Rad54, Rad55 and Rad57. All these genes were initially identified as being defective in the repair of damaged DNA caused by ionizing radiation and dysfunctional mutants in these genes were subsequently shown to be deficient in both genetic recombination and the recombinational repair of DNA lesions (Game, Radiation sensitive mutants and repair in yeast. Yeast Genetics: Fundamental and applied aspects. J.F.T. Spencer and A.R.W. Smith, eds (New-York: Springer-Verlag):109-137 (1983); Haynes, et al., DNA repair and mutagenesis in yeast. The molecular biology of the yeast *Saccaromyces cerevisiae*: life cycle and inheritance. J.N. Strathern, E.W. Jones and J.M. Broach eds (Cold Spring

Harbor, New York: Cold Spring Harbor Laboratory press):371-414 (1981); Resnick, Investigating the genetic control of biochemical events in meiotic recombination. P.B. Moens, ed. (New York: Academic Press):157-210 (1987)). Recent experiments found that homozygous knock-outs of Rad51 in chicken B cells are extremely sensitive to radiation, accumulate double-stranded DNA breaks, and undergo programmed cell death (Sonoda, et al., *EMBO* **17**:598-608 (1998)).

Although Rad51 RNA transcripts and protein are present in all cell types, the highest transcript levels are in tissue active in recombination, including spleen, thymus, ovary and testis (Morita, et al., *Proc. Natl. Acad. Sci. USA* **90**:6577-6580 (1993)). For example, Rad51 is specifically induced in murine B cell nuclei undergoing Ig class switch recombination (Li, et al., *Proc. Natl. Acad. Sci. USA* **93**:10222-10227 (1996)), Rad51 is enriched in the synaptonemal complexes which join paired homologous chromosomes in spermatocytes undergoing meiosis (Haaf, et al., *Proc. Natl. Acad. Sci. USA* **92**:2298-2302 (1995); Ashley, et al., *Chromosoma* **104**:19-28 (1995); Plug, et al., *Proc. Natl. Acad. Sci. USA* **93**:5920-5924 (1996)), and Rad51 nuclear localization changes dramatically in response to DNA damage in cultured cell lines, when multiple discreet foci are re-distributed in the nucleus and stain vividly with anti-Rad51 antibodies (Haaf, et al., *Proc. Natl. Acad. Sci. USA* **92**:2298-2302 (1995).

Targeted disruption of Rad51 leads to an embryonic lethal phenotype in mouse and the dying embryo cells are very sensitive to radiation (Tsuzuki, et al., *Proc. Natl. Acad. Sci. USA* **93**:6236-6240 (1996); Lim & Hasty, *Mol. Cell. Biol.* **16**:7133-7143 (1996)). Attempts to generate viable homozygous $rad51^{-/-}$ embryonic stem cells have not been successful. These results show that Rad51 plays an essential role in cell proliferation, a surprise in view of the viability of *S. cerevisiae* carrying Rad51 deletions. It is also interesting that Rad51 is associated with RNA polymerase II transcription complexes (Maldonado, et al., *Nature* **381**:86-89 (1996). Although the specificity and functional nature of these interactions are not clear, these observations taken together point to a pleiotropic role for human Rad51 in DNA metabolism (repair, recombination, transcription), and maintenance of genomic integrity.

Human Rad51 protein interacts directly with wild type p53 protein, and the regions necessary for this interaction have been mapped (Sturzbecher et, al., *EMBO* **15**:1992-2002 (1996); Buchhop, et al., *Nucleic Acids Res* **25**:3868-3874 (1997)). Rad51 interacts with two different regions of p53 (amino acids 94-160 and 264-315), and p53 interacts with the region between amino acids 125 and 220 of Rad51. This latter region is necessary for homo-oligomerization of Rad51. Therefore, p53 may inhibit Rad51 activity by blocking the formation of active Rad51 oligomers. Furthermore, p53 inhibits Rad51 ATPase and DNA strand exchange activities. Interestingly, p53 mutants often found in cancer cells, are reported to bind Rad51 less efficiently than wild type 53 and fail to inhibit its biochemical activities. Taken together, known interactions between Rad51 and p53 suggest that 1) in normal cells p53 interacts with and downregulates Rad51 and 2) in tumor cells with p53 mutations, unregulated Rad51 could possibly lead to uncontrolled recombination, genetic instability, and radiation resistance by upregulating DNA recombination and DNA repair (Sturzbecher, et al., *EMBO* **15**:1992-2002 (1996); Ohnishi, et al., *Biochem. Biophys. Res. Comm.* **245**:319-324 (1998).

Rad51 also interacts with BRCA1 and BRCA2 (Scully, et al., *Cell* **88**:265-275 (1997); Sharan, et al., *Nature* **386**:804-810 (1997)). Inherited mutations in BRCA1 cause familial breast and ovarian cancer, and inherited mutations in BRCA2 case familial breast cancer (Wooster, et al., *Science* **265**:2088-2090 (1994); Smith, et al., *Nature Genet.* **2**:128-131 (1992); Easton, et al., *Am. J. Hum. Genet.* **52**:678-701 (1993); Gayther, et al., *Nature Genet.* **15**:103-105 (1997)). Sharan, et al., *J. Nature.* **386**:804-810 (1997)) showed that BRCA2 binds to Rad51 and that mouse BRCA2 knockouts are both early embryonic lethal and hypersensitive to radiation, similar to Rad51 knockout mice. Furthermore, certain BRCA2 peptides bind Rad51 and inhibit cell growth. Scully, et al., *Cell* **88**:265-275 (1997) showed that BRCA1 binds to Rad51 and co-localizes with it in synaptonemal complexes.

Recently, several human members of the Rad51 family of related genes have been identified, including Rad51B (Albala, et al., *Genomics* **46**:476-479 (1997), Rad51C (Dosanjh, et al., *Nucleic Acids Res* **26**:1179-1184 (1998)), Rad51D (Pittman, et al., *Genomics* **49**:103-111 (1998)), XRCC2 (Cartwright et al., *Nucleic Acids Res* **26**:3084-

3089-793 (1998)) and XRCC3 (Liu, et al., *Mol Cell* **1**:783 (1998)). While these genes are homologous to human Rad51, it is also possible that they are related to certain other members of the Rad52 epistasis group such as Rad55 and Rad57. The chromosomal locations of all these genes have been mapped. XRCC2 maps to chromosome 7q36.1, a region associated with radiation resistance in human glial tumors.

The c-Abl tyrosine kinase is activated by ionizing radiation and other DNA damaging agents. It was recently reported that cAbl constitutively interacts with Rad51 and phosphorylates Rad51 on Tyr-54. Thus, ionizing radiation induces c-Abl-dependent phosphorylation of Rad51. Furthermore, phosphorylation of Rad51 by c-Abl inhibits Rad51 binding to DNA and its function in ATP-dependent DNA strand exchange (Yuan, et al., *J. Biol. Chem.* **273**:3799-3802 (1998)).

Accordingly, it is desirable to inhibit Rad51 for treatment of Rad51 related disorders, and to potentiate radiation therapy by inhibiting double-strand break repair. It is further desirable to downregulate Rad51 to sensitize human tumor cells to chemotherapeutic agents like cisplatin.

SUMMARY OF THE INVENTION

Provided herein are methods and compositions for inhibiting Rad51 and human Rad51 homologues. The methods can be performed in vitro or in vivo and have a number of applications as further described below including the treatment of cancer. Also provided are compositions and related methods of using Rad51 inhibitors.

In one aspect of the invention, a small molecule is used to inhibit Rad51 or a human Rad51 homologue. In one embodiment, the biological or biochemical activity of Rad51 or a human Rad51 homologue is inhibited wherein the biological or biochemical activity is selected from the group of DNA dependent ATPase activity, formation of Rad51 foci, nucleic acid strand exchange, DNA binding, filament formation, DNA pairing and DNA repair, wherein said DNA repair is double stranded break repair, single stranded annealing or post replication recombinational repair.

The small molecule can be any one of or a combination of small molecules including but not limited to a nucleotide diphosphate, a nucleotide analogue, a DNA minor groove binding drug, a xanthine or a xanthine derivative such as caffeine.

In one aspect of the invention, the nucleotide diphosphate is selected from the group consisting of ADP, GDP, CDP, UDP and TDP.

In another aspect of the invention, the nucleotide analogue is a nucleotide diphosphate complexed with aluminum fluoride. The nucleotide diphosphate complexed with aluminum fluoride can be selected from, for example, the group consisting of ADP.AlF4, GDP.AlF4, CDP.AlF4, UDP.AlF4 and TDP.AlF4.

In another embodiment, the nucleotide analogue is a non-hydrolyzable nucleotide. For example, the non-hydrolyzable nucleotide can be ATPγS, GTPγS, UTPγS, CTPγS, TTPγS, ADPγS, GDPγS, UDPγS, CDPγS, TDPγS, AMPγS, GMPγS, UMPγS, CMPγS, TMPγS, ATP-PNP, GTP-PNP, UTP-PNP, CTP-PNP, TTP-PNP, ADP-PNP, GDP-PNP, UDP-PNP, CDP-PNP, TDP-PNP, AMP-PNP, GMP-PNP, UMP-PNP, CMP-PNP, or TMP-PNP

In one aspect of the invention, the Rad51 or human Rad51 homologue is a DNA minor groove binding drug which can be selected from the group consisting of distamycin, netropsin, bis-benzimidazole and actinomycin. Alternatively, the inhibitor is a peptide.

Inhibiting Rad51 and/or human Rad51 homologues has a number of applications. In one method, a Rad51,or human Rad51 homologue, inhibitor is combined with a composition comprising Rad51 and components required for Rad51 activity such cell such as DNA and ATP. The composition can be a solution in a test tube for an in vitro assay. In another embodiment, the Rad51 and human Rad51 homologue inhibitors are administered to a cell. The cell can be a cancerous cell. In one embodiment, the cell is of an individual with cancer.

Also provided herein is a method for inducing sensitivity to radiation and DNA damaging chemotherapeutics in an individual comprising administering to said individual a composition comprising a Rad51, or human Rad51 homologue, inhibitor, wherein said inhibitor is a small molecule. In a preferred embodiment, the individual has cancer.

In yet a further aspect of the invention, a method for inducing apoptosis is provided. In one embodiment, the method comprises administering a Rad51, or human Rad51 homologue, inhibitor to a cell. The cell can be cancerous or of an individual with cancer.

The small molecule Rad51 or human Rad51 homologue inhibitors provided herein can be administered in a pharmaceutical acceptable carrier. In one embodiment the inhibitors are further administered in conjunction with a ligand which targets the cells in need thereof. In a preferred embodiment, the ligand targets cancerous cells. The ligand can be an antibody which targets cell surface ligands which are specific to diseased cells such as cancer cells or cells infected with HIV.

In yet a further aspect of the invention, a fragment of Rad51 or a human Rad51 homologue is provided wherein said fragment consists essentially of a binding site for a small molecule, wherein said small molecule regulates the biological or biochemical activity of Rad51. In a preferred embodiment, the small molecule inhibits Rad51 activity. In another embodiment, the site is the p53 binding site on Rad51 or the human Rad51 homologue. The Rad51 homologue can be selected from the group consisting of Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3.

Other aspects of the invention will be apparent from the detailed description provided below.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a series of discoveries relating to the pivotal role that Rad51 plays in a number of cellular functions, including those involved in disease states. In particular, described herein are compositions and methods for inhibiting Rad51 and

methods of treatment for disease states associated with Rad51 activity as further defined below using Rad51 inhibitors. Other compositions and methods related to Rad51 inhibition are also described.

Accordingly, in one aspect of the invention, a method for inhibiting at least one Rad51 biological or biochemical activity is provided. The method comprises administering a Rad51 inhibitor to a composition comprising Rad51. The composition can be an in vitro solution comprising Rad51 and Rad51 inducers such as DNA and ATP under conditions which allow Rad51 activity. In one embodiment, the composition is a cell. In a preferred embodiment, the Rad51 inhibitor is a small molecule.

Rad51 biological or biochemical activity as used herein can be selected from the group consisting of DNA dependent ATPase activity, formation of Rad51 foci, nucleic acid strand exchange, DNA binding, nucleoprotein filament formation, DNA pairing and DNA repair. DNA repair and recombination are generally considered biological activities. DNA repair can be double stranded break repair, single stranded annealing or post replication recombinational repair.

As further described below, in another aspect of the invention, a Rad51 inhibitor inhibits cell proliferation. In a further aspect also described below, a Rad51 inhibitor results in the cells containing it to be more sensitive to radiation and/or chemotherapeutic agents. In yet another aspect, a Rad51 inhibitor induces apoptosis as further described below.

In one aspect, a Rad51 inhibitor or an agent or composition having Rad51 inhibitory activity is defined herein as an agent or composition inhibiting expression or translation of a Rad51 nucleic acid or the biological activity of a Rad51 peptide by at least 30%, more preferably 40%, more preferably 50%, more preferably 70%, more preferably 90%, and most preferably by at least 95%. In one embodiment herein, a Rad51 inhibitor inhibits expression or translation of a Rad51 nucleic acid or the activity of a Rad51 protein by 100%. In one aspect, inhibition is defined as any detectable decrease in Rad51 activity compared to a control not comprising the Rad51 inhibitor.

In one embodiment, Rad51 inhibitors can include inhibitors of Rad51 homologues such as RecA and/or inhibitors that sensitize cells to radiation and also affect aspects of recombination *in vivo,* which were not previously known to inhibit Rad51. Thus, in one embodiment, Rad51 as used herein refers to Rad51 and its homologues, preferably human homologues. In one embodiment, Rad51 excludes non-human homologues. Rad51 homologues include RecA and Rad51 homologues in yeast and in mammals. Genes homologous to E. coli RecA and yeast Rad51 have been isolated from all groups of eukaryotes, including mammals. Morita, et al., *PNAS USA* **90**:6577-6580 (1993); Shinohara, et al., *Nature Genet.* **4**:239-243 (1993); Heyer, *Experentia,* **50**:223-233 (1994); Maeshima, et al., *Gene* **160**:195-200 (1995). Human Rad51 homologues include Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3. Albala, et al., *Genomics* **46**:476-479 (1997); Dosanjh, et al., *Nucleic Acids Res* **26**:1179(1998); Pittman, et al., *Genomics* **49**:103-11 (1998); Cartwright, et al., *Nucleic Acids Res* **26**:3084-3089 (1998); Liu, et al., *Mol Cell* **1**:783-793 (1998). In preferred embodiments, Rad51 inhibitors provided herein were not previously known to inhibit RecA or other Rad51 homologues and were not known to induce sensitizing of cells to radiation. In one embodiment, Rad51 as used herein excludes homologues thereof.

The Rad51 inhibitor can inhibit Rad51 directly or indirectly, preferably directly by interacting with at least a portion of the Rad51 nucleic acid or protein. Additionally, the inhibitors herein can be utilized individually or in combination with each other.

In a preferred embodiment, the small molecule is preferably 4 kilodaltons (kd) or less. In another embodiment, the small molecule is less than 3 kd, 2kd or 1kd. In another embodiment the small molecule is less than 800 daltons (D), 500 D, 300 D, 200 D or 100 D.

In one embodiment, the Rad51 inhibitor is an inorganic or organic molecule. In a preferred embodiment, the Rad51 inhibitor is a small organic molecule, comprising functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically will include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The Rad51 inhibitor may

comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more chemical functional groups. As further discussed below, Rad51 inhibitors can comprise nucleotides, nucleosides, and nucleotide and nucleoside analogues. Nucleotides as used herein refer to XYP, wherein X can be U, T, G, C or A (base being uracil, thymine, guanine, cytosine or adenine, respectively), and Y can be M, D or T (mono, di or tri, respectively). In another embodiment, nucleotides can include xathanine, hypoxathanine, isocytosine, isoguanine, etc. Analogues as used herein includes derivatives of and chemically modified nucleotides and nucleosides. In one embodiment, methyl methanesulfonate is excluded.

In one aspect of the invention, the Rad51 inhibitor is a nucleotide diphosphate. In a preferred embodiment, the Rad51 inhibitor is selected from the group consisting of ADP, GDP, CDP, UDP and TDP. In preferred embodiments, ADP is excluded.

In another aspect of the invention, the Rad51 inhibitor is a nucleotide analogue. In a preferred embodiment, the Rad51 inhibitor is a nucleotide diphosphate complexed with aluminum fluoride. In one embodiment, the Rad51 inhibitor is selected from the group consisting of ADP.AlF4, GDP.AlF4, CDP.AlF4, UDP.AlF4 and TDP.AlF4.

In yet a further aspect of the invention, the Rad51 inhibitor is a non-hydrolyzable nucleotide. In a preferred embodiment, the Rad51 inhibitor is selected from the group consisting of ATPγS, GTPγS, UTPγS, CTPγS, TTPγS, ADPγS, GDPγS, UDPγS, CDPγS, TDPγS, AMPγS, GMPγS, UMPγS, CMPγS, TMPγS, ATP-PNP, GTP-PNP, UTP-PNP, CTP-PNP, TTP-PNP, ADP-PNP, GDP-PNP, UDP-PNP, CDP-PNP, TDP-PNP, AMP-PNP, GMP-PNP, UMP-PNP, CMP-PNP, and TMP-PNP In preferred embodiments, ADPγS is excluded.

Also another embodiment, the Rad51 inhibitor is a DNA minor groove binding drug. In a preferred embodiment, the Rad51 inhibitor is selected from the group consisting of distamycin, netropsin, bis-benzimidazole and actinomycin.

In yet another embodiment, the Rad51 inhibitor is a peptide. By "peptide" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradations.

The peptides can be naturally occurring or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. Thus, procaryotic and eukaryotic proteins can be Rad51 inhibitors. Rad51 inhibitors may also be peptides from bacterial, fungal, viral, and mammalian sources, with the latter being preferred, and human proteins being especially preferred.

In a preferred embodiment, the Rad51 inhibitors are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence.

Preferred peptides include but are not limited to amino acids 94-160 and 264-315 of p53 and fragments of Rad51 antibodies.

In a preferred embodiment, the Rad51 inhibitors are nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., *Tetrahedron* **49**(10):1925 (1993)) and references therein; Letsinger, *J. Org. Chem.* **35**:3800 (1970); Sprinzl et al., *Eur. J. Biochem.* **81**:579 (1977); Letsinger et al., *Nucl. Acids Res.* **14**:3487 (1986); Sawai et al, *Chem. Lett.* **805** (1984), Letsinger et al., *J. Am. Chem. Soc.* **110**:4470 (1988); and Pauwels et al., *Chemica Scripta* **26**:141 91986)), phosphorothioate (Mag et al., *Nucleic Acids Res.* **19**:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., *J. Am. Chem. Soc.* **111**:2321 (1989)), O-methylphophoroamidite linkages (see Eckstein, oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, *J. Am. Chem. Soc.* **114**:1895 (1992); Meier et al., *Chem. Int. Ed. Engl.* **31**:1008 (1992); Nielsen, *Nature* 365:566 (1993); Carlsson et al., *Nature* **380**:207 (1996)), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., *Proc. Natl. Acad. Sci. USA* **92**:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., *Angew. Chem. Intl.* Ed. English **30**:423 (1991); Letsinger et al., *J. Am. Chem. Soc.* **110**:4470 (1988); Letsinger et al., *Nucleoside & Nucleotide* **13**:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., *Bioorganic & Medicinal Chem. Lett.* **4**:395 (1994); Jeffs et al., *J. Biomolecular NMR* **34**:17 (1994); *Tetrahedron Lett.* **37**:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., *Chem. Soc. Rev.* pp169-176 (1995)). Several nucleic acid analogs are described in Rawls, *C &*

E News p. 35 (June 2, 1997). All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs including PNA can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc.

In one aspect it is understood that Rad51 inhibitors may bind to Rad51, but exclude agents which generally activate Rad51 such as DNA on which Rad51 normally binds to in the process of recombinational activity, ATP, etc.

As generally for proteins, nucleic acid Rad51 inhibitors may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eucaryotic genomes may be used as is outlined above for proteins.

Rad51 inhibitors are obtained from a wide variety of sources, as will be appreciated by those in the art, including libraries of synthetic or natural compounds. Any number of techniques are available for the random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications to produce structural analogs.

In a preferred embodiment, the methods include both in vitro and in vivo applications, preferably in vivo. Accordingly, in a preferred embodiment, the methods comprise the steps of administering a Rad51 inhibitor to a sample comprising Rad51 under physiological conditions, preferably to a cell.

The cell that the Rad51 inhibitor is administered to may be a variety of cells. Preferably the cell is mammalian, and preferably human. The cell may be any cell in a site in need of Rad51 inhibition such as diseased cells including cancerous cells and cells infected with viruses such as HIV as further discussed below.

Administration may occur in a number of ways. The addition of the Rad51 inhibitor to a cell will be done as is known in the art for other inhibitors, and may include the use of nuclear localization signal (NLS). NLSs are generally short, positively charged (basic) domains that serve to direct the entire protein in which they occur to the cell's nucleus. Numerous NLS amino acid sequences have been reported including single basic NLS's such as that of the SV40 (monkey virus) large T Antigen (Pro Lys Lys Lys Arg Lys Val), Kalderon (1984), et al., *Cell* **39**:499-509; the human retinoic acid receptor-ß nuclear localization signal (ARRRRP); NFκB p50 (EEVQRKRQKL; Ghosh et al., *Cell* **62**:1019 (1990); NFκB p65 (EEKRKRTYE; Nolan et al., *Cell* **64**:961 (1991)); and others (see for example Boulikas, *J. Cell. Biochem.* **55**(1):32-58 (1994)), hereby incorporated by reference) and double basic NLS's exemplified by that of the Xenopus (African clawed toad) protein, nucleoplasmin (Ala Val Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys Lys Leu Asp), Dingwall, et al., *Cell* **30**:449-458, 1982 and Dingwall, et al., *J. Cell Biol.*, **107**:641-849; 1988). Numerous localization studies have demonstrated that NLSs incorporated in synthetic peptides or grafted onto reporter proteins or other molecules not normally targeted to the cell nucleus cause these molecules to be concentrated in the nucleus. See, for example, Dingwall, and Laskey, *Ann, Rev. Cell Biol.* **2**:367-390, 1986; Bonnerot, et al., *Proc. Natl. Acad. Sci. USA* **84**:6795-6799, 1987; Galileo, et al., *Proc. Natl. Acad. Sci. USA* **87**:458-462, 1990.

There are a variety of techniques available for introducing a Rad51 inhibitor into cells. The techniques vary depending upon whether the inhibitor is transferred into cultured

cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of inhibitors into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau et al., *Trends in Biotechnology* **11**:205-210 (1993)). Special or other liposomes, modified electroporation, chemical treatment or Piezo injection techniques are particularly preferred.

The inhibitory agents may be administered in a variety of ways, orally, systemically, topically, parenterally e.g., subcutaneously, intraperitoneally, intravascularly, etc. In one embodiment, the inhibitors are applied to the site of a tumor (or a removed tumor) intra-operatively during surgery. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. Generally, a therapeutic amount for the need is used, for example, to achieve inhibition of cellular proliferation, radiation or chemotherapeutic sensitization or inducing apoptosis.

The Rad51 inhibitory molecules can be combined in admixture with a pharmaceutically or physiologically acceptable carrier vehicle. Therapeutic formulations are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

The pharmaceutical compositions can be prepared in various forms, such as granules, aerosols, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

In some situations it is desirable to provide the inhibitor with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., *J. Biol. Chem.* **262**:4429-4432 (1987); and Wagner et al., *Proc. Natl. Acad. Sci. USA* **87**:3410-3414 (1990).

Dosages and desired drug concentrations of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary physician. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti, J. and Chappell, W. "The use of interspecies scaling in toxicokinetics" In Toxicokinetics and New Drug Development, Yacobi *et al.*, Eds., Pergamon Press, New York pp. 42-96 (1989).

In a preferred embodiment, the methods comprise identifying the inhibitory effect of the Rad51 inhibitor. For example, determining the effect on double strand break repair, homologous recombination, sensitivity to ionizing radiation, class switch recombination,

cellular inhibition, induction of apoptosis, etc. Assays are detailed in Park, *J. Biol. Chem.* **270**(26):15467 (1995) and Li et al., *PNAS USA* **93**:10222 (1996), Shinohara et al., *supra*, (1992), all of which are hereby incorporated by reference. Further assays are discussed below in the examples.

In an embodiment provided herein, the invention provides methods of treating disease states requiring inhibition of cellular proliferation. In a preferred embodiment, the disease state requires inhibition of at least one of Rad51 expression, translation or the biological activity of Rad51 as described herein. As will be appreciated by those in the art, a disease state means either that an individual has the disease, or is at risk to develop the disease.

Disease states which can be treated by the methods and compositions provided herein include, but are not limited to hyperproliferative disorders. More particular, the methods can be used to treat, but are not limited to treating, cancer (further discussed below), premature aging, autoimmune disease, arthritis, graft rejection, inflammatory bowel disease, proliferation induced after medical procedures, including, but not limited to, surgery, angioplasty, and the like. Thus, in one embodiment, the invention herein includes application to cells or individuals afflicted or impending affliction with any one of these disorders.

The compositions and methods provided herein are particularly deemed useful for the treatment of cancer including solid tumors such as skin, breast, brain, cervical carcinomas, pancreas, testicular carcinomas, etc. More particularly, cancers that may be treated by the compositions and methods of the invention include, but are not limited to: Cardiac: sarcoma (angiosarcoma, fibrosarcoma, rhabdomyosarcoma, liposarcoma), myxoma, rhabdomyoma, fibroma, lipoma and teratoma; Lung: bronchogenic carcinoma (squamous cell, undifferentiated small cell, undifferentiated large cell, adenocarcinoma), alveolar (bronchiolar) carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesothelioma; Gastrointestinal: esophagus (squamous cell carcinoma, adenocarcinoma, leiomyosarcoma, lymphoma), stomach (carcinoma, lymphoma, leiomyosarcoma), pancreas (ductal adenocarcinoma, insulinoma, glucagonoma,

gastrinoma, carcinoid tumors, vipoma), small bowel (adenocarcinoma, lymphoma, carcinoid tumors, Karposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma, fibroma), large bowel (adenocarcinoma, tubular adenoma, villous adenoma, hamartoma, leiomyoma); Genitourinary tract: kidney (adenocarcinoma, Wilm's tumor [nephroblastoma], lymphoma, leukemia), bladder and urethra (squamous cell carcinoma, transitional cell carcinoma, adenocarcinoma), prostate (adenocarcinoma, sarcoma), testis (seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors, lipoma); Liver: hepatoma (hepatocellular carcinoma), cholangiocarcinoma, hepatoblastom, angiosarcoma, hepatocellular adenoma, hemangioma; Bone: osteogenic sarcoma (osteosarcoma), fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma (reticulum cell sarcoma), multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma (osteocartilaginous exostoses), benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors; Nervous system: skull (osteoma, hemangioma, granuloma, xanthoma, osteitis deformans), meninges (meningioma, meningiosarcoma, gliomatosis), brain (astrocytoma, medulloblastoma, glioma, ependymoma, germinoma [pinealoma], glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma, congenital tumors), spinal cord neurofibroma, meningioma, glioma, sarcoma); Gynecological: uterus (endometrial carcinoma), cervix (cervical carcinoma, pre-tumor cervical dysplasia), ovaries (ovarian carcinoma [serous cystadenocarcinoma, mucinous cystadenocarcinoma, unclassified carcinoma], granulosa-thecal cell tumors, Sertoli-Leydig cell tumors, dysgerminoma, malignant teratoma), vulva (squamous cell carcinoma, intraepithelial carcinoma, adenocarcinoma, fibrosarcoma, melanoma), vagina (clear cell carcinoma, squamous cell carcinoma, botryoid sarcoma [embryonal rhabdomyosarcoma], fallopian tubes (carcinoma); Hematologic: blood (myeloid leukemia [acute and chronic], acute lymphoblastic leukemia, chronic lymphocytic leukemia, myeloproliferative diseases, multiple myeloma, myelodysplastic syndrome), Hodgkin's disease, non-Hodgkin's lymphoma [malignant lymphoma]; Skin: malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Karposi's sarcoma, moles dysplastic nevi, lipoma, angioma, dermatofibroma, keloids, psoriasis; and Adrenal glands: neuroblastoma. Thus, the term

"cancerous cell" as provided herein, includes a cell afflicted by any one of the above identified conditions.

The individual, or patient, is generally a human subject, although as will be appreciated by those in the art, the patient may be animal as well. Thus other animals, including mammals such as rodents (including mice, rats, hamsters and guinea pigs), cats, dogs, rabbits, farm animals including cows, horses, goats, sheep, pigs, etc., and primates (including monkeys, chimpanzees, orangutans and gorillas) are included within the definition of patient. In a preferred embodiment, the individual requires inhibition of cell proliferation. More preferably, the individual has cancer or a hyperproliferative cell condition.

The compositions provided herein may be administered in a physiologically acceptable carrier to a host, as previously described. Preferred methods of administration include systemic or direct administration to a tumor cavity or cerebrospinal fluid (CSF).

In one aspect, the Rad51 inhibitors herein induce sensitivity to alkylating agents, DNA cross-linkers, intra and inter strand, cisplatin and related compounds and radiation. Induced sensitivity (also called sensitization or hypersensitivity) is measured by the cells tolerance to radiation or alkylating agents. For example, sensitivity, which is measured, i.e., by toxicity, occurs if it is increased by at least 20%, more preferably at least 40%, more preferably at least 60%, more preferably at least 80%, and most preferably by 100% to 200% or more.

In an embodiment herein, the methods comprising administering the Rad51 inhibitors provided herein further comprise administering an alkylating agent or radiation. For the purposes of the present application the term ionizing radiation shall mean all forms of radiation, including but not limited to alpha, beta and gamma radiation and ultra violet light, which are capable of directly or indirectly damaging the genetic material of a cell or virus. The term irradiation shall mean the exposure of a sample of interest to ionizing radiation, and term radiosensitive shall refer to cells or individuals which display unusual adverse consequences after receiving moderate, or medically acceptable (i.e., nonlethal

diagnostic or therapeutic doses), exposure to ionizing irradiation. Alkylating agents include BCNU and CCNU. Additionally, radiation sensitizers (e.g., xanthine and xanthine derivatives including caffeine) can be applied with, before or after the Rad51 inhibitors.

In one embodiment herein, the Rad51 inhibitors provided herein are administered to prolong the survival time of an individual suffering from a disease state requiring the inhibition of the proliferation of cells. In a preferred embodiment, the individual is further administered radiation or an alkylating agent.

In yet another aspect of the invention, a fragment of Rad51 is provided wherein said fragment consists essentially of a binding site for a small molecule, wherein said small molecule regulates the biological or biochemical activity of Rad51. Preferably, the regulation is inhibitory. In one embodiment, the binding site is the binding site for p53.

Generally, the binding site is identified by combining the inhibitor with fragments of Rad51. In one embodiment, the fragments are from between amino acids 125 and 220. In one embodiment, Rad51 125-220 is fragmented to fragments of 5-25 amino acids and then tested separately or in random recombinations to determine the binding site by standard binding techniques.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are specifically incorporated by reference in their entirety.

EXAMPLES

Example 1

Rad51 Biochemical Assays Compatible for High Throughput Screening

Several assays are available to detect the activity of Rad51. Strand exchange reactions catalyzed by human Rad51 are monitored with oligonucleotide substrates. These substrates are very convenient and easy to use because of machine synthesis and labeling of oligonucleotides either with fluorophores or with biotin. Rad51 protein carries out strand exchange in three distinct phases: I) presynapsis, during which Rad51 protein binds cooperatively and stoichiometrically to single-stranded DNA and forms a right handed helical nucleoprotein filament; II) synapsis, in which duplex DNA is taken up into the nucleoprotein filament and homologously aligns; and III) DNA strand displacement, which produces a recombinant (heteroduplex) double-stranded DNA molecule and a displaced single-stranded DNA .

Intermediates of the reaction known as joint molecules (also referred to as D-loops) and the final products are monitored either by filter assays, fluormeters, or by gel electrophoresis.

A given inhibitor can inactivate Rad51 by interfering with any one of these three steps. Hence, test Rad51 inhibitors are added at the stage of presynapsis, synapsis or strand displacement stage of DNA strand exchange. These methods are used to determine whether the inhibitors are acting by a) interfering with the cooperative polymerization of Rad51 on single-stranded DNA, b) affecting the pairing of the filament to the homologous DNA target or, c) affecting the process of strand exchange by inhibiting hydrolysis of ATP.

Filter binding DNA strand exchange assays (solid phase-based) of Rad51 activity which are compatible with high throughput screening. Filter binding assays are based on single-stranded DNA binding to nitrocellulose membranes under the appropriate salt conditions. The linear duplex DNA is labeled with bases linked to fluorophores, ^{32}P, or

biotin and the single-stranded DNA substrates are unlabeled. After uptake of the double-stranded DNA into the nucleoprotein filament, DNA base pair switching displaces the complementary strand of the parental duplex DNA. As a result of DNA strand displacement, hybrid DNA intermediates of the DNA strand exchange reaction contain single-stranded DNA tails, and one of the products of strand exchange is single-stranded DNA; both of which are trapped on nitrocellulose filters. The unreacted linear double-stranded DNA cannot bind to the membrane and is washed away in the filtrate. Since the initial single-stranded DNA used to make the nucleoprotein filament is not labeled, it is not detected.

The filter binding assays are easy to use and extremely reliable. These assays are performed in high throughput mode, for example, using a 96 well format on manifolds fitted with nitrocellulose membranes.

Fluorescence spectroscopy-based assays for monitoring the DNA strand exchange activity of human Rad51 are highly specific and compatible with high throughput screening. Assays based on fluorescence to measure DNA pairing and DNA strand exchange by human Rad51 protein have been developed. This approach enables one to distinguish homologous DNA pairing from subsequent DNA strand exchange. Homologous pairing of a single-stranded oligonucleotide with a duplex oligonucleotide is measured by fluorescence resonance energy transfer (FRET). Energy transfer between two fluorescent dyes indicates their proximity. In the case of DNA, the proximity of two complementary strands labeled with dyes are determined by FRET. For example, when a 5'-Watson strand labeled with fluorescein comes into proximity with a complementary 3'-Crick strand labeled with rhodamine, the overlap between its emission spectrum and the excitation spectrum of rhodamine allows the nonradiative transfer of energy to rhodamine by fluorescence resonance energy transfer (Bazemore et al., *J. Biological Chem.* **272**(23):14672-14682 (1997) and Bazemore, et al., *PNAS USA* **94**:11863-11868 (1997)).

Homologous DNA pairing assay by FRET. A test oligonucleotide labeled at its 3' end with fluorescein is used to form the nucleoprotein filament with Rad51. Rhodamine is

attached to the 5' end of the complementary strand in duplex DNA. Homologous pairing between the two DNA molecules juxtaposes the two fluorescent molecules, resulting in nonradiative energy transfer from fluorescein to rhodamine when fluorescein is excited at 493 nm, near its excitation peak. As a result of the energy transfer, the fluorescence emission from fluorescein is quenched and that from rhodamine is enhanced.

To measure homologous pairing of a test single-stranded oligonucleotide with its homologous duplex oligonucleotide, an 83-mer oligonucleotide (minus strand) labeled at its 3' end with fluroescein is preincubated with 1.2 μM Rad51 protein in a reaction mixture containing 1 mM $MgCl_2$, 25 mM HEPES (pH 7.4), 1 mM DTT, 2 mM ATP and 100 μg of BSA per ml for 4 minutes at 37°C. The concentration of $MgCl_2$ is increased to 30 mM, and finally 3 μM duplex DNA (labeled with rhodamine at the 5' end of the plus strand) is added.

DNA strand exchange assay by FRET. When the fluorescein and rhodamine are juxtaposed by 20 Å on opposite complementary strands, the emission from the fluorescein is quenched and that from rhodamine is enhanced as a result of energy transfer. To measure the strand exchange activity, both fluorophores are present in the duplex where they are juxtaposed. When strand exchange is completed, the two labeled strands are separated from each other as monitored by the enhanced emission from fluorescein.

To measure DNA strand exchange by FRET, Rad51 protein is added to unlabeled single-strand oligonucleotide for 4 min. at 37°C followed by the addition of the filament to a reaction mixture containing 30 mM $MgCl_2$ and 3 μM duplex oligonucleotide (labeled on the 3' end of the minus strand with fluorescein and on the 5' end of the plus strand with rhodamine). The final concentrations of ssDNA and protein are 3 μM and 1.2 μM, respectively.

Fluorescence emission spectra are recorded from 502 to 620 nm upon excitation at 493 nm on an SLM 8000C (SLM Aminco, Urbana, IL) or similar spectrofluorimeter.

Example 2

Determination of Lead Compound Specificity

As discussed above, inhibitors for Rad51 biological activity are confirmed in a number of ways. The following assays are used to assay for a change in biological activity to initially identify inhibitors, or to determine the specificity of identified inhibitors: D-loop assay, DNA dependent ATPase assay, nucleoprotein filament assay, and complementary single-strand hybridization assay. These assays are unique features of the Rad51 protein and determine the specificity of, for example, small molecules that inhibit Rad51 protein activity.

D-loop assay. The non-enzymatic uptake of a homologous single-stranded DNA by a negatively supercoiled DNA leads to the formation of a DNA displacement loop (D-loop, Figure 12) (Holloman et al., *PNAS, USA* **76**:1638-1642 (1975)).

Rad51 enzymes both catalyze D-loop formation under physiological conditions. Negative superhelicity is not required in these reactions catalyzed by RecA or Rad51. Only members of RecA and Rad51 protein families can catalyze the formation of D-loops under physiological conditions.

Several standard assays monitor D-loop formation by DNA recombination enzymes. Most commonly used assays use duplex DNA (either supercoiled DNA or a linear duplex DNA) and a linear single-stranded DNA (either an oligonucleotide or a relatively longer linear single-stranded DNA) as substrates. The D-loop products are analyzed either by filter assays or by gel electrophoresis. Filter assays are simple, fast and samples are analyzed in a plate format. In these experiments, the target duplex DNA is labeled and the single-stranded DNA substrate is unlabeled. After uptake of the single-stranded DNA into duplex DNA, the tails of the unincorporated single-stranded DNA of the hybrid molecules are trapped on the filter and only the D-loops are detected. Unreacted double-stranded DNA (superhelical DNA or linear duplex DNA) do not bind to the membrane. If necessary, the D-loop products can also be monitored by following the separation of hybrids by gel electrophoresis.

DNA dependent ATPase assay. The ATP binding domain is highly conserved throughout evolution in the homologues of RecA protein (Heyer, *Experentia* **50**:223-233 (1994)). The unique feature of the ATPase activity of RecA and Rad51 is that this activity is DNA dependent. Rad51 hydrolyzes ATP only in the presence of single-stranded DNA and has no ATPase activity in the absence of DNA.

To monitor ATP hydrolysis, labeled ATP is incubated with single-stranded DNA and Rad51. The reaction mixture is incubated at 37°C for 30 min. and an aliquot of the reaction is applied directly onto CEL 300 PEI/UV_{254} thin layer chromatography plates to separate the product of hydrolysis (ADP) from the substrates. Non-DNA dependent ATPases are used as controls for these reactions. Small molecule compounds that inhibit the ATPase activity of Rad51 would not be expected to affect the activity of other ATPase enzymes.

Assay of nucleoprotein filament formation. Rad51 protein binds cooperatively to DNA to form a right-handed helix. The resulting protein-DNA complex is an active nucleoprotein filament which catalyzes DNA pairing and DNA strand exchange reactions. The DNA helix inside the filament is extended 1.5 times the size of B-form duplex length. This structure of the nucleoprotein filament is a hallmark feature of RecA and Rad51 proteins and is DNA sequence independent. The DNA inside the filament is completely protected from phosphodiesterases, as RecA and Rad51 proteins bind to and protect the phosphate backbone from cleavage. Formation of nucleoprotein filaments is easily monitored by protein-based filter binding assays and fluorescent dye displacement assays. Another DNA binding protein is used as a control.

Complementary single-strand DNA renaturation assay. Rad51 protein promotes the hybridization of complementary strands of DNA under specific conditions in which the spontaneous renaturation of complementary strands does not occur. Hybridization activity is easy to monitor in a DNA micro array format or by filter binding assays. Other single-strand annealing proteins, such as SSB, are used as controls.

Example 3

Cytotoxicity and Cell Growth Assays and Sensitization of Human Tumor Cells to Radiation and DNA-damaging Chemicals

A sulforhodamine B-based optical density assay of protein in cultured cells is used as a cell-based high throughput drug screen for inhibitors of Rad51 activity. The phenotypes screened for are cytotoxicity and growth inhibition in target tumor (breast, brain and prostate) and control (non tumorigenic) cell lines. Cells are placed in 96-well microtiter plates. Next, these drugs are introduced after one day of culture, and treat for an additional 96 hours. Assays begin at the beginning of drug treatment, at 48 hours and at 96 hours. Qualitative changes are monitored by comparing the amount of cellular protein present at the beginning of the drug incubation period with the amount of protein present in control and test cultures at day 3 and day 5 of growth. Other time points are added as necessary. Quantitative drug-induced changes in culture growth are evaluated using the doubling time and fractional growth rate. See, Skehan, *Assays of Cell Growth and Cytotoxicity*, G. Studzinski, ed., 2nd Ed., pp 169-191 (1995); Skehan, et al., *J. Natl. Cancer Inst.* **82**:1107-12 (1990); Skehan, et al., Cell Biol. Toxicol. **2**:357-368 (1986).

Also determined is Rad51 activity in these samples using methods outlined above to determine whether the biological effects measured are specific to alterations in Rad51 activity. This is done by comparing the results to antisense oligodeoxynucleotides that specifically down-regulate Rad51 protein.

Sensitization of human tumor cells to radiation and DNA-damaging chemicals. Following DNA damage, cells may survive by undergoing transient cell arrest and regrowth, or may remain in permanent cell cycle arrest; or the cells may simply die. The extent to which Rad51 down-regulation sensitizes cells to DNA damage is determined by assaying Rad51 down-regulation shifts on the dose response curves for DNA damaging agents (radiation, BCNU, cyclophosphamide, cis-platin) in systems that measure growth, survival and death. The effect of inhibition on the cell cycle is monitored by looking for cell cycle arrest in G1 or G2 phase by FACS technology. The effects on cell growth are screened using the sulforhodamine B assay. Assayed is the effect on cell survival using

a clonigenic assay to determine the surviving fraction of clonigenic cells. Apoptosis is assayed using either a flow cytometric assay for subdiploid fractions or by using the TUNEL method, which utilizes terminal deoxynucleotide transferase to incorporate fluorescein-conjugated deoxyuridine triphosphate into DNA nicks formed in apoptotic cells. As noted above, parallel experiments with antisense oligodeoxynucleotides assure the specificity.

Example 4

Human Tumor Xenografts Implanted in Nude Mice

Human tumors in a human host and human tumors transplanted into athymic mice respond similarly to antineoplastic drugs, and therefore xenografts grown in athymic mice provide an invaluable preclinical model to validate Rad51 inhibitors identified in cell-based assays.

There are two aims to these experiments. One is to determine whether Rad51 down-regulation has a growth inhibitory effect. The second evaluates Rad51 down-regulation as a sensitizer to DNA damage. The experimental blueprint for these studies in human tumor xenografts is: a) define growth curves for the untreated xenograft and for xenografts treated with inhibitors of Rad51. This aim also determines how growth is related to Rad51 down-regulation; b) choose appropriate doses of DNA damaging agents that allow us to compare the responses of the control and Rad51 down-regulated cell lines; c) quantitate the amount of sensitization to DNA damage.

Implantation and growth of human tumor xenografts in nude mice. The target tumor cell lines chosen herein can all form tumors in nude mice. 1×10^5, 1×10^6, and 1×10^7 cells taken from cell culture in the log phase of growth are injected into the flanks of nude mice, and the tumor measured daily for 50 days, until the tumor reaches $2000mm^3$, or until the animal becomes debilitated. Each group has 5 mice. Generally tumors are treated when they reach a volume of $50\text{-}100mm^3$.

Determination of lethal dose. This is a simple determination of lethality of the lead compound found by escalating single doses in mice which are indexed to intracellular concentrations that down-regulate Rad51 in culture. Toxicity is investigated by physical exam and organ histopathology.

Treatment of tumors with lead compounds. Once tumors reach a treatable size, animals are injected with escalating doses of the lead compound. Both systemic and local routes of administration are tested. The tumors are examined for Rad51 activity and for volume. Toxicity of the compound in these animals are assayed by weight gain, serum chemistries and organ histopathology (including liver, lung, kidneys, heart, gastrointestinal tract and brain).

Dose finding for DNA damage. The growth characteristics of the cell lines are established and then various doses of DNA damaging agents (for example, 0, 5, 10 and 15 Gy for radiation) are used to define a series of growth curves that describe response. This information allows selection of doses to compare control cell lines to the Rad51 down-regulated cell lines. Each group has at least 5 animals and appropriately matched controls.

Sensitization of radiation and chemotherapy treatments by lead compounds. Control and Rad51 down-regulated cell lines are grown and treated with DNA damaging agents at times and doses determined above. If the growth rates of control and down-regulated lines are similar, direct comparisons are made between growth delays caused by radiation in sets of modified and unmodified cell lines. However, if their growth rates differ, a dose is chosen that will allow us to measure the average size of tumors at a specific point in time. When the difference between the average sizes of control (i.e., not down-regulated) tumors treated and not treated with DNA damaging agents is ΔU and the difference between the average sizes of down-regulated tumors treated and not treated with DNA damaging agents is ΔM, then $\Delta U - \Delta M > 0$. Studies include 4 groups of 15 animals for each lead compound. Assuming a normal distribution, this provides 90% power to detect a difference $\Delta U - \Delta M$ of 1.5 times the standard deviation, assuming a one-tailed hypothesis test is $\alpha = 0.05$.

Other references specifically incorporated by reference are Haaf, T., *Pharmac. Ther.* ***65***:19-46 (1995); Haaf, T., and Schmid,M. *Exp. Cell Res.* **192**:325-332 (1991); and Owaga, et al, *Science* **259**:1896-1899 (1993).

WE CLAIM:

1. A method for inhibiting Rad51 or a human Rad51 homologue comprising contacting an inhibitor of Rad51 or an inhibitor of a human Rad51 homologue with Rad51 or a human Rad51 homologue to inhibit a Rad51 biological or biochemical activity, wherein said inhibitor is a small molecule.

2. A method for inhibiting cell proliferation, said method comprising administering a Rad51 inhibitor or human Rad51 homologue inhibitor to a cell, wherein said inhibitor is a small molecule.

3. A method for inducing sensitivity to radiation and DNA damaging chemotherapeutics in an individual comprising administering to said individual a composition comprising a Rad51 inhibitor or human Rad51 homologue inhibitor, wherein said inhibitor is a small molecule.

4. A method for inducing apoptosis in a cell, said method comprising administering to said cell a composition comprising a Rad51, or human Rad51 homologue, inhibitor, wherein said inhibitor is a small molecule.

5. The method of claim 1, 2, 3 or 4 wherein said biological or biochemical activity is selected from the group of DNA dependent ATPase activity, formation of Rad51 foci, nucleic acid strand exchange, DNA binding, filament formation, DNA pairing and DNA repair, wherein said DNA repair is double stranded break repair, single stranded annealing or post replication recombinational repair.

6. The method of claim 1, 2, 3 or 4 wherein said small molecule is selected from the group consisting of a nucleotide diphosphate, a nucleotide analogue, a DNA minor groove binding drug, a xanthine and a xanthine derivative.

7. The method of claim 6 wherein said xanthine derivative is caffeine.

8. The method of claim 6 wherein said nucleotide diphosphate is selected from the group consisting of ADP, GDP, CDP, UDP and TDP.

9. The method of claim 6 wherein said nucleotide analogue is selected from the group consisting of a nucleotide diphosphate complexed with aluminum fluoride and a non-hydrolyzable nucleotide.

10. The method of claim 9 wherein said nucleotide diphosphate complexed with aluminum fluoride is selected from the group consisting of ADP.AlF4, GDP.AlF4, CDP.AlF4, UDP.AlF4 and TDP.AlF4.

11. The method of claim 9 wherein said non-hydrolyzable nucleotide is selected from the group consisting of ATPγS, GTPγS, UTPγS, CTPγS, TTPγS, ADPγS, GDPγS, UDPγS, CDPγS, TDPγS, AMPγS, GMPγS, UMPγS, CMPγS, TMPγS, ATP-PNP, GTP-PNP, UTP-PNP, CTP-PNP, TTP-PNP, ADP-PNP, GDP-PNP, UDP-PNP, CDP-PNP, TDP-PNP, AMP-PNP, GMP-PNP, UMP-PNP, CMP-PNP, and TMP-PNP

12. The method of claim 3 wherein said DNA minor groove binding drug is selected from the group consisting of distamycin, netropsin, bis-benzimidazole and actinomycin.

13. The method of claim 1, 2, 3 or 4 wherein said small molecule is a peptide.

14. Use of a small molecule inhibitor of Rad51 or a human Rad51 homologous in the formulation of a medicament for use in inhibiting a biological or biochemical activity of Rad51.

15. The use of claim 14 wherein said biological or biochemical activity is selected from the group of DNA dependent ATPase activity, formation of Rad51 foci, nucleic acid strand exchange, DNA binding, filament formation, DNA pairing and DNA repair, wherein said DNA repair is double stranded break repair, single stranded annealing or post replication recombinational repair.

16. The use of claim 14 wherein said small molecule inhibitor is selected from the group consisting of a nucleotide diphosphate, a nucleotide analogue, a DNA minor groove binding drug, a xanthine and a xanthine derivative.

17. The use of claim 16 wherein said xanthine derivative is caffeine.

18. The use of claim 16 wherein said nucleotide diphosphate is selected from the group consisting of ADP, GDP, CDP, UDP and TDP.

19. The use of claim 16 wherein said nucleotide analogue is selected from the group consisting of a nucleotide diphosphate complexed with aluminum fluoride and a non-hydrolyzable nucleotide.

20. The use of claim 19 wherein said nucleotide diphosphate complexed with aluminum fluoride is selected from the group consisting of ADP.AlF4, GDP.AlF4, CDP.AlF4, UDP.AlF4 and TDP.AlF4.

21. The use of claim 19 wherein said non-hydrolyzable nucleotide is selected from the group consisting of ATPγS, GTPγS, UTPγS, CTPγS, TTPγS, ADPγS, GDPγS, UDPγS, CDPγS, TDPγS, AMPγS, GMPγS, UMPγS, CMPγS, TMPγS, ATP-PNP, GTP-PNP, UTP-PNP, CTP-PNP, TTP-PNP, ADP-PNP, GDP-PNP, UDP-PNP, CDP-PNP, TDP-PNP, AMP-PNP, GMP-PNP, UMP-PNP, CMP-PNP, and TMP-PNP.

22. The use of claim 16 wherein said DNA minor groove binding drug is selected from the group consisting of distamycin, netropsin, bis-benzimidazole and actinomycin.

23. The use of claim 14 wherein said inhibiting is in a cancerous cell.

24. The use of claim 14 wherein said small molecule inhibitor is a peptide.

25. A pharmaceutical composition comprising a small molecule inhibitor of Rad51 or a human Rad51 homologue selected from the group consisting of a nucleotide

diphosphate, a nucleotide analogue, a DNA minor groove binding drug, a xanthine and a xanthine derivative and a pharmaceutically acceptable carrier.

26. The composition of claim 25 wherein said xanthine derivative is caffeine.

27. The composition of claim 25 wherein said nucleotide diphosphate is selected from the group consisting of ADP, GDP, CDP, UDP and TDP.

28. The composition of claim 25 wherein said nucleotide analogue is selected from the group consisting of a nucleotide diphosphate complexed with aluminum fluoride and a non-hydrolyzable nucleotide.

29. The composition of claim 28 wherein said nucleotide diphosphate complexed with aluminum fluoride is selected from the group consisting of ADP.AlF4, GDP.AlF4, CDP.AlF4, UDP.AlF4 and TDP.AlF4.

30. The composition of claim 28 wherein said non-hydrolyzable nucleotide is selected from the group consisting of ATPγS, GTPγS, UTPγS, CTPγS, TTPγS, ADPγS, GDPγS, UDPγS, CDPγS, TDPγS, AMPγS, GMPγS, UMPγS, CMPγS, TMPγS, ATP-PNP, GTP-PNP, UTP-PNP, CTP-PNP, TTP-PNP, ADP-PNP, GDP-PNP, UDP-PNP, CDP-PNP, TDP-PNP, AMP-PNP, GMP-PNP, UMP-PNP, CMP-PNP, and TMP-PNP.

31. The composition of claim 25 wherein said DNA minor groove binding drug is selected from the group consisting of distamycin, netropsin, bis-benzimidazole and actinomycin.

32. A fragment of Rad51 or a human Rad51 homologue wherein said fragment consists essentially of a binding site for a small molecule, wherein said small molecule regulates the biological or biochemical activity of Rad51.

33. The fragment of claim 32 wherein regulates is inhibitory.

34. The fragment of claim 32, wherein p53 binds to said site.

35. The fragment of claim 32, wherein said biological or biochemical activity is selected from the group of DNA dependent ATPase activity, formation of Rad51 foci, nucleic acid strand exchange, DNA binding, filament formation, DNA pairing and DNA repair, wherein said DNA repair is double stranded break repair, single stranded annealing or post replication recombinational repair.

36. The fragment of claim 32 wherein said small molecule is selected from the group consisting of a nucleotide diphosphate, a nucleotide analogue, a DNA minor groove binding drug, a xanthine and a xanthine derivative.

37. The fragment of claim 32 wherein said DNA minor groove binding drug is selected from the group consisting of distamycin, netropsin, bis-benzimidazole and actinomycin.

38. The fragment of claim 32 wherein said small molecule is a peptide.

39. The fragment of claim 32 wherein said fragment is of Rad51.

40. The fragment of claim 32 wherein said fragment is of Rad51B.

41. The fragment of claim 32 wherein said fragment is of Rad51C.

42. The fragment of claim 32 wherein said fragment is of Rad51D.

43. The fragment of claim 32 wherein said fragment is of XRCC2.

44. The fragment of claim 32 wherein said fragment is of XRCC3.